YERSINIA IN MUNICIPAL GROUND WATER SUPPLIES IN SOUTHERN ONTARIO

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YERSINIA IN MUNICIPAL GROUND WATER SUPPLIES

IN SOUTHERN ONTARIO

by

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SUMMARY

Raw and treated well water supplies in Southern Ontario were examined for the presence of Yersinia species. Organisms identified as Y. enterocolitica were isolated, using an enrichment technique, in only 0.9% of the raw ground water supplies analysed; none were serologically similar to strains associated with human disease. Yersinia species, none confirmed to be Y. enterocolitica, were isolated sporadically from other supplies. Results show that the total coliform indicator may not flag the presence of Yersinia species.

INTRODUCTION

Yersinia enterocolitica (Y. enterocolitica) has been recovered from patients suffering from gastro-intestinal disease and a variety of other forms of illness (1). Swine are a reservoir of the common human serotype O:3 (2), and Y. enterocolitica and Y. enterocolitica-like organisms are frequently recovered from raw (unpasteurized) milk (3), raw meats (4), oysters (5) and vegetables used in salads (6). The organisms have been isolated from surface waters in the USA (7) and Canada (8), and have been found in both treated and untreated water supplies in Washington State, USA (9). Y. enterocolitica has been isolated from private wells in various locations throughout Ontario (10), and occurs with some frequency in well waters in Northern Ontario (11).

This study was primarily to determine if Y. enterocolitica organisms could be isolated from samples of municipal ground water in Southern Ontario as readily as had been the case in Northern Ontario (Thunder Bay Region). Raw and treated water samples from well supplies, submitted to the Central Laboratory of the Ministry of the Environment, were examined using the same method employed in the Thunder Bay Regional Laboratory. Subsequently, methods were modified with the use of a newly-available selective medium and also by an enrichment technique to enhance the recovery of Y. enterocolitica. Isolates of Y. enterocolitica were submitted to the "National Reference Service for Y. enterocolitica and Y. pseudotuberculosis" (NRS) of the Ontario Ministry of Health for confirmation and further testing.

MATERIALS AND METHODS

ISOLATION METHODS

A. Direct Isolation Method

1. Source of Isolates. P-A bottles (12) and/or membrane filters (13) were used for the isolation of Y. enterocolitica, after being inoculated with raw or treated water samples submitted to the Central Laboratory and

examined for the presence of total and fecal coliforms and other indicators.

a. P-A Bottles. All P-A bottles, showing the absence of coliforms or other specified indicators, but showing a slight acid reaction and/or heavy bacterial growth after five days of incubation at 35°C were subcultured onto modified MacConkey medium (14) (MacConkey agar (BBL) plus 0.2 gm/L $CaCl_2$ and 10 gm/L Tween 80 (J.T. Baker)). This procedure differed from the isolation method used in the Thunder Bay laboratory only in the use of the modified MacConkey medium in place of regular MacConkey agar.

Later in the study, a new selective medium for the isolation of Y. enterocolitica, developed by Schiemann (15) became available; this medium, CIN medium from Difco, was then used in addition to the modified MacConkey medium, to isolate Y. enterocolitica from the P-A cultures.

b. Membrane Filter Cultures. Dark red (1) or dew-drop-like (10) colonies were picked off m-Endo LES membrane cultures, after 24 hours of incubation at 35°C and following the counting of coliform colonies, and inoculated onto modified MacConkey medium as in 1a) above.

2. Selection of Colonies. After 24 hours of incubation at 35°C on modified MacConkey medium, all colourless to pale pink colonies (14) less than 3mm in diameter were transferred to nutrient agar (Difco) for a further 24 hours at 35°C for purification.

Flat colonies with deep red confined centres (15) or a pink colouration after 24 or 48 hours of incubation at 35°C on CIN agar, were similarly purified.

B. Enrichment Isolation Method

1. Source of Isolates. During the final period of the study, isolations of Y. enterocolitica were attempted using an enrichment method after that used by Wetzler (16). The remaining portions of raw ground water samples from a given supply, after the samples had been processed for indicator organisms by Central Laboratory staff and following storage for one week at 4°C, were pooled to provide a "composite" sample of approximately 150 mL of that source water. "Composite" samples obtained in this fashion were prepared from the same well water supplies for which the examination of single samples inoculated into P-A bottles or MF cultures had been completed earlier. The results of routine bacteriological analysis of the water samples making up the "composites" were also recorded.

 The 150 mL "composite" sample was filtered through a 0.45 μ membrane filter (Gelman) using normal procedures. The filter was then carefully rolled with sterile forceps and inserted into a large (25 mL) culture tube containing 10 mL cooked meat medium (Difco). These membrane cultures were incubated in the refrigerator at 4°C for two weeks and then plated onto CIN agar. Negative membrane cultures were reincubated, with further plating at three and four weeks of incubation, before being discarded.

2. Selection of Colonies. Colonies typical of Y. enterocolitica after incubation for 24 hours at 35°C on CIN agar were subcultured onto trypticase soy agar (BBL) at 35°C for 24 hours for purification.

IDENTIFICATION OF Y. ENTEROCOLITICA

Purified cultures on the nutrient or trypticase soy agar plates were tested for cytochrome-oxidase production by transferring a loopful of growth to a filter paper impregnated with one percent tetramethyl-p-phenylene diamine dihydrochloride. All oxidase negative cultures were screened by inoculating a urea agar slant (Difco) incubated at 35°C and two tubes of

trypticase soy broth (BBL), one incubated at 35°C and the other at 25°C; two tubes of trypticase soy agar were also inoculated at this time and incubated at 25°C.

All urea positive cultures showing motility by wet mount preparation after incubation at 25°C but not after incubation at 35°C were retained for further identification, and the two trypticase soy agar slants were then stored under refrigeration; urea negative isolates showing the typical Y. enterocolitica motility characteristics were also retained, subsequent to discovering that some isolates showing negative reactions on the Difco urea medium could be positive on other urea medium. All other cultures were discarded.

Further identification was performed on one of the trypticase soy agar slant cultures using the API-20E (Analytab Products) system for Enterobacteriaceae, according to manufacturer's directions. These directions specified incubation of the inoculated API-20E strips at 35°C. It was determined, however, that incubation at this temperature could result in misidentification, with other Yersinia species appearing as Y. enterocolitica; incubation at 25°C produced identification results equivalent to those obtained by NRS using conventional biochemicals incubated at 25°C. After submission of the first five cultures to NRS for confirmation had revealed this discrepancy, all subsequent Y. enterocolitica identifications on API-20E were confirmed at 25°C, when test strips were available.

CONFIRMATION OF Y. ENTEROCOLITICA

The trypticase soy agar slants from cultures identified by API-20E as Y. enterocolitica, which had been incubated at 25°C for 24 hours and subsequently held under refrigeration, were forwarded to NRS of the Ontario Ministry of Health. The cultures were biotyped, using standard biochemicals rather than the API-20E diagnostic system. Cultures exhibiting biochemical reactions typical of Y. enterocolitica were serologically typed using standard techniques.

RESULTS

A) Direct Isolation Method

Of 2811 P-A bottles examined by inoculation onto modified MacConkey medium, only 15 showed the presence of Yersinia species. The results of both API-20E identification and the corresponding confirmed results from NRS are shown in Table 1. None of the isolates was confirmed as Y. enterocolitica.

Subcultures from P-A bottles onto CIN medium yielded no Yersinia isolations from 118 samples examined. When organisms were isolated on this medium, they tended to be the same as those isolated on modified MacConkey medium inoculated in parallel. However, CIN medium appeared to be more selective in that a higher proportion of the inoculated plates showed no growth.

No Yersinia isolations were obtained by picking colonies from the membrane filters used for coliform analysis. Although the technique had been used with some success by others, it was not pursued past the early stages of this study because of problems with overgrowth. Membrane filters from 36 water samples only, were examined before this method was abandoned.

B) Enrichment Isolation Method

Following enrichment of "composite" samples, eight of 544 were positive for Yersinia species. As shown in Table 2, one was confirmed as Y. kristensenii, two were identified as Y. intermedia and the remaining five were confirmed by NRS as Y. enterocolitica.

NRS carried out serological testing of these five Y. enterocolitica strains and the results are shown in Table 3. Two of the strains proved to be biotype 1 and serologically untypeable, two were confirmed as biotype 3 and serologically untypeable. Only one isolate of biotype 1 could be serotyped and was determined to be serotype O:14. This serotype has been previously isolated from lake water (5), and is not one that appears to have been associated with human infections.

TABLE 1. Yersinia Isolations from P-A Bottle Cultures of Various Water Samples Inoculated onto Modified MacConkey Medium at 35°C.

Location	Source Type*	Identification API-20E @ 35°C	Identification API-20E @ 25°C	National Reference Service
Fenelon Falls	TG	Y. enterocolitica	ND**	Y. intermedia
Acton	RG	Y. fredricksenii	ND	Y. intermedia
Barrie	RG	Y. enterocolitica	ND	Y. intermedia
Waterloo	PS	Y. enterocolitica	ND	Y. kristensenii
Cambridge	RG	Y. enterocolitica	Y. intermedia	Y. intermedia
Kitchener	DS	Y. enterocolitica#	ND	ND
Barrie	RG	Y. intermedia##	ND	ND
Kitchener	DS	Y. enterocolitica#	Y. enterocolitica#	ND
Cambridge	RS	Y. enterocolitica	ND	Y. kristensenii
Caledon	RG	Y. fredricksenii	Y. intermedia	Y. intermedia
Caledon	RG	Y. enterocolitica	Y. intermedia	Y. intermedia
Kitchener	RG	Y. ruckeri##	ND	ND
Caledon	RG	Y. enterocolitica	No Growth	No Growth
Niagara	RG	Y. fredricksenii	ND	Y. intermedia
Kitchener	RG	Y. enterocolitica	ND	Y. fredricksenii

* TG = treated ground, RG = raw ground, PS - pumping station, DS = distribution system, RS = reservoir

** ND = not done

\# very low selectivity)

\## questionable identification) from the API-20E

TABLE 2. Yersinia Isolations from "Composite" Samples of Well Waters Using the Enrichment Method.

Location	Identification API-20E @ 35°C	Identification API-20E @ 25°C	National Reference Service
Nobleton	Y. kristensenii	Y. kristensenii	Y. kristensenii
Caledon(1)	Y. enterocolitica	Y. enterocolitica	Y. enterocolitica
Caledon(2)	Y. enterocolitica	Y. enterocolitica	Y. enterocolitica
Caledon (3)	Y. enterocolitica	Y. enterocolitica	Y. enterocolitica
Caledon(4)	ND	Y. intermedia	ND
McLeod	Y. enterocolitica	Y. enterocolitica	Y. enterocolitica
Mono Mills	ND*	Y. intermedia	ND
Courtland	Y. enterocolitica	Y. enterocolitica	Y. enterocolitica

*not done

TABLE 3. Types of Y. enterocolitica Isolated by Enrichment from Well Waters.

Location	Biotype	Serotype
Caledon(1)	1	Non-typeable
Caledon(2)	1	Non-typeable
Caledon(3)	3	Non-typeable
McLeod	1	O:14
Courtland	3	Non-typeable

Results of the routine bacteriological analysis of the samples used to prepare the "composites" from which Yersinia species were isolated are shown in Table 4. In one of the "composites" (McLeod), positive for Y. enterocolitica, coliforms and other indicators were absent, and the standard plate count was only 170 organisms per mL. At Nobleton, coliforms were absent from the two samples making up the "composites" but Y. kristensenii was isolated. The Caledon (3) "composite" showed only low numbers of coliforms (2 per 100 mL) in one of the samples yet the presence of Y. enterocolitica was confirmed. In the composite from Mono Mills, made up from four well samples, anaerogenic coliforms were reported present in one sample, and Y. intermedia was isolated. Coliforms were present in the P-A test in only one of the samples making up the "composite" of Courtland well water, and Y. enterocolitica was isolated. In composites (1) and (2) from Caledon, containing Y. enterocolitica, coliforms were present in all the samples making up the "composites"; whereas, in the Caledon (4) "composites", from which Y. intermedia was recovered, coliforms and fecal coliforms were present in two of the constituent samples.

DISCUSSION

Investigations of Y. enterocolitica in rivers, reservoirs and private wells in Colorado, confirmed its presence in about 17% of river and reservoir samples and 1.8% of wells; among isolates only 6% consisted of serotypes known to cause disease in man (17). Other workers (7), (8), (9), (10) have reported similar findings. The health significance of the presence of Y. enterocolitica in drinking water supplies is still largely unknown, but it has been associated with a few outbreaks of waterborne disease (18) (19).

Very few of the P-A cultures (the majority from chlorinated, distribution system water samples) examined in this study showed the presence of Yersinia species, and none were confirmed to be Y. enterocolitica. It is acknowledged that incubation of the P-A MacConkey broth for up to five

TABLE 4. Results of Routine Bacteriological Analysis of Samples Used to Prepare "Composites" which Contained Yersinia Species.

Location	Pooled Samples	P-A Results*	MF - Results Total Coliforms per 100 mL	Standard Plate Count (48hr) per mL
Nobleton	Well 2)	CA	ND##	ND
	Well 3)	neg#	ND	ND
Caledon(1)	Well 1)	CP	ND	ND
	Well 2)	CP, FC, FS	2482**	450
Caledon(2)	Well 1)	CP	0	46
	Well 2)	ND	30	150
	Well 2A)	ND	30**	190
Caledon(3)	Well 1)	neg	0	0
	Well 2)	CA, AE, FS	2	56
	Well 3)	CA	0	>2400
Caledon(4)	Well 1)	CA	0	2
	Well 2)	CP, FC	148	43
	Well 2A)	CP, FC	100	46
	Well 3)	CA	ND	100
McLeod	Well 1)	CA	0	170
	Well 2)	CA	ND	ND
Mono Mills	Well 2)	neg	ND	ND
	Well 3)	CA	ND	ND
	Well 4)	CA	ND	ND
	Well 1)	AC	ND	ND
Courtland	Well 1)	CP	ND	ND
	Well 2)	CA	ND	ND
	Well 3)	neg	ND	ND

* CP = coliforms present, FC = fecal coliforms present, FS = fecal streptococci present, AE = aeromonas species present, AC = anaerogenic coliforms present, CA = coliforms absent

** approximate counts

ND = not done

neg = PA test negative, no growth

days at 35°C may not provide optimum conditions for the recovery of Y. enterocolitica. However, the Thunder Bay laboratory appeared to be successful in the recovery of Yersinia species using essentially similar methodology. The results reported here show that Yersinia species do occur in various ground water supplies in Southern Ontario.

For the major part of the study, only those P-A bottles which had shown an absence of indicator bacteria, but the presence of growth and/or a weak acid reaction were examined for Yersinia. It is possible that Y. enterocolitica organisms could have been present in P-A bottles showing the presence of coliform bacteria and other indicators. However, Wetzler (16) showed that in chlorinated supplies, Yersinia tended to occur more frequently in samples where coliforms were absent or present only in low numbers; in another report by Wetzler, it was stated that there was no evidence of the total coliform indicator being useful for flagging the likelihood of the presence of Yersinia (20). It could be inferred, therefore, that if Yersinia organisms were present in chlorinated supplies, they would be just as likely to occur in coliform-negative P-A cultures as in coliform-positive ones.

Isolates of Yersinia species from enriched, composite samples taken from raw ground water supplies were few in number, resulting in only a 1.5% recovery rate. It is interesting to note that, for all the Yersinia cultures isolated by the enrichment method, the API-20E test at 35°C and 25°C, and the NRS test methods all yielded the same identification (Table 2). For cultures isolated from the P-A bottles on modified MacConkey medium, the identification by the API-20E test at 25°C and/or the NRS test methods, did not correspond with that from the API-20E test at 35°C (Table 1). The reason for this was not determined, but might be related to the different isolation media and technique used. Y. enterocolitica was found on three occasions where at least one of the samples in the composite apparently contained high numbers

of coliforms, but also on one occasion where coliforms were very low in number (2 per 100 mL). The organism was found on one occasion where no coliforms were detected. One "composite" showing the presence of Y. intermedia, contained a sample with anaerogenic coliforms and fecal coliforms. The samples in the "composite" containing Y. kristensenii did not contain coliforms. These results appear to support Wetzler's views.

The use of CIN medium for 'plating out' greatly simplified the recovery of Yersinia species, because of suppression of other organisms which had grown in the P-A cultures, but there is insufficient data to conclude definitely that the recovery of the organism was superior.

It appears that for the differentiation of Y. enterocolitica from other Yersinia species, it may be preferable to incubate the API-20E strip at 25°C and not at 35°C as directed. Limited observations with duplicate strips incubated at both temperatures showed that incubation at 25°C did not compromise the other biochemical reactions in the test strip. For Yersinia isolates from the direct isolation method, standard biochemical test results differed from some of those obtained on the API-20E system. All isolates were citrate negative by API-20E, whereas standard biochemical tests showed a utilization of citrate. Additionally, different results were obtained for some sugars, in particular rhamnose and melibiose; the test result for these sugars is important in determining different relatedness groups within the Yersinia genus. The incubation temperature is apparently critical, and workers have recommended that biochemical testing, in particular the carbohydrate utilization tests, be carried out at 25°C to 26°C (6) (21) (22). When incubated at 35°C the API-20E results for both melibiose and rhamnose were negative, resulting in an identification of Y. enterocolitica; when the API-20E test strip was incubated at 25°C, these sugars both gave positive results, for an identification of Y. intermedia; this result was confirmed by NRS (23). However, the API-20E test system was incubated routinely at

35°C at the Thunder Bay laboratory for the identification of Y. enterocolitica (11), and all of the cultures sent to NRS were confirmed as Y. enterocolitica. Further, Yersinia isolates by the enrichment method did not produce results on API-20E incubated at 35°C, which differed from those produced at 25°C or by the NRS system. The apparent difference in biochemical response may be related to the isolation technique used.

The use of an enrichment technique for isolation in the final phases of this study, involves some problems in the interpretation of results. Other than "to show they are there" (24), the value of detecting Y. enterocolitica only after enrichment has revealed the presence of very low numbers, would be highly debatable. Schiemann (25) has stated; "It is apparent that our food and water supplies frequently contain Y. enterocolitica and that mere isolation of an organism identifiable as Y. enterocolitica is not sufficient basis for condemning nor implicating any vehicle in an outbreak of gastro-enteritis, without accompanying human culture or serological evidence. The ubiquitous distribution of organisms which are biochemically variable but accepted as the same species further complicates a judgement regarding the significance of isolation from the environment". In fact, Wetzler (16) in a post-presentation comment has stated an unwillingness to accept an illness as being caused by Yersinia unless an increase in antibody titre to the organism can be demonstrated in the patient. The presence of Y. enterocolitica in the intestinal contents may only reflect that food (chicken liver, cheddar cheese, watercress etc.) consumed previously, contained the organisms. Reports of gastrointestinal illness attributed to Yersinia species, based only on the isolation of the organism from fecal material, must be regarded as questionable.

The etiology of Y. enterocolitica appears to resemble that of other "opportunistic pathogens", such as Klebsiella pneumoniae, Pseudomonas aeruginosa and Legionella pneumophila, which can also be found in low

densities in the natural environment and occasionally as contaminants in waters from distribution systems. Further accumulation of reliable epidemiological information is required to clarify the role of these organisms in human disease.

CONCLUSIONS

Organisms identified as Y. enterocolitica were isolated from various ground water supplies in Southern Ontario; none were serologically similar to strains normally associated with human disease. There were insufficient isolations to enable any associations between Y. enterocolitica and any particular type of ground water supply to be made. The results of this study, and those of other workers, indicate that Y. enterocolitica is likely to be found in all water supplies, and that total coliforms and other indicator bacteria may not flag its presence.

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